Contents

MEMORANDUM

SUBJECT: Ecological Hazard Assessment for TERA R-19-0001

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I. INTRODUCTION

EPA has received a TSCA Environmental Release Application (TERA) from Synthetic Genomics, Inc. (SGI) to test one intergeneric eukaryotic algal construct, *Parachlorella* sp. STR26155, for field trial in open ponds.

The introduced intergeneric DNA gene present in the final construct encodes for TurboGFP). The gene is regulated by the endogenous *ACP1* (Acyl Carrier Protein) promoter and terminator of *Parachlorella*. The expression of TurboGFP will be used by SGI to specifically track the STR26155 strain in open-culture and in the environment.

The selectable marker gene *ble* (for resistance against bleomycin family antibiotics; e.g., zeocin) was also used during intermediate cloning steps, but was removed from the final subject strain via Cre-lox recombinase technology, leaving only a short, non-coding 34bp loxP site as the other intergeneric component. These intergeneric additions to create the subject strain, *Parachlorella* sp. STR26155, resulted in no discernable phenotypic differences relative to the recipient strain STR00012.

The aim of this TERA and the research for which it seeks authorization is, in part, to establish baseline environmental conditions in and around the test facility, and to evaluate and confirm the sufficiency of control and monitoring equipment and techniques developed for this and other similar outdoor R&D programs. This TERA also aims to lay the foundations necessary to link the biology work in the lab with successful scale-up in the field by experimenting at a manageable scale. Gaining insight into how algal strains (top candidates today as well as those to be developed) perform in industrially-relevant settings will inform the design of the technology and ultimately accelerate its development and deployment. It will also reduce the risk of failure that comes with continuing to design a technology without knowing the conditions and constraints it will ultimately face at-scale. The submitter hopes that this effort will contribute to the development of a globally-relevant Safety, Health & Environment

package, or "template", for subsequent TERA and MCAN (TSCA Microbial Commercial Activity Notification) submissions to U.S. EPA and international environmental protection agencies.

II. TAXONOMY AND CHARACTERIZATION OF MICROORGANISM

A. Recipient Microorganism

The submitter identifies the parental organism as a wild-type *Parachlorella* sp. (SGI strain designation - STR00010). This strain was isolated from seawater samples collected by SGI near the Hawaiian island of Oahu. *Parachlorella* sp. STR00010 was then subjected to UV mutagenesis to create STR00012, the recipient strain for this TERA, which has higher biomass productivity than STR00010. The taxonomic identity of the recipient, *Parachlorella* sp. STR00012, was verified by SGI using 18S rRNA and ITS data. There are currently two species of *Parachlorella, P. kessleri,* and *P. beijerinckii*. A third species, *P. hussii* has been proposed Bock et al. (2011) but has uncertain taxonomic status. However, SGI's particular isolate STR00010 could not be assigned to any of these species, so the taxonomic designation is *Parachlorella* sp. The phylogenetic analyses provided by the submitter were confirmed in the Taxonomic Identification Report for R-19-0001 (Strope, 2019).

1. The Genus

The *Parachlorella* genus has not been assessed by EPA in other submissions. The closely-related *Chlorella* genus however, have been accessed in two previous TERA applications (R-17-0002, R-18-0001). Both *Chlorella* and *Parachlorella* are taxonomically classified in the Class Trebouxiophyceae and under the Family Chlorellaceae (Huss et al., 2009). Due to the many similarities in morphology and physiology with microalgae within this class and family, many coccoid green algal (termed 'green ball') groups were previously misclassified under the genus *Chlorella* (Krienitz et al., 2004). As taxonomic identification moved towards more modern molecular phylogenetic approaches (e.g., utilizing sequences of 18S rRNA and ITS2 regions), the genus *Chlorella* was broken up into more distinct genera, one of which being *Parachlorella* (Krienitz et al., 2004). In light of this historical misclassification and recent reclassification within the Chlorellaceae Family, many studies and work done with green microalgae fitting the previous "*Chlorella*" description, are likely applicable to the genus *Parachlorella*.

The *Chlorella* genus was first delineated by Beyerinck in 1890. A comprehensive description of the genus *Chlorella* was first addressed by Shihira and Krauss (1965), in response to the lack of a sound taxonomic framework from which to base the identity of over 41 isolates known at the time. In 1976, Kessler identified 77 strains across 12 taxa, based on physiological and biochemical properties. Since then the genus has been found to have few useful diagnostically morphological characteristics, making it difficult to identify under a light microscope alone, and only through more rigorous methods can it be clearly classify as belonging to a specific species (i.e., DNA analysis) (Bock et al., 2011; Zou et al., 2016). Therefore, a more robust framework, based on polyphasic taxonomic approaches, has been developed to describe well over 100 potentially different *Chlorella* species (Bock et al., 2011; Zou et al., 2016). Based on integrative or polyphasic taxonomy a new system has been established which differs completely from the traditional artificial system of *Chlorella* and its relatives based on morphology alone. With the introduction of chemotaxonomy to *Chlorella* and other taxa our understanding of the taxonomy of *Chlorella* has changed radically. Based on SSU- and ITS rDNA sequences and light microscopy observations, various publications have demonstrated how the high level of cryptic diversity found within *Chlorella*; and the polyphyletic characters between *Chlorella* and *Dictyosphaerium*, has

resulted in numerous taxonomic revisions of these organisms (Zou et al., 2016). For example, Bock et al. (2011) detected six lineages of *Dictyosphaerium*-like strains that are closely related to *Chlorella vulgaris* and described several new species. Krienitz et al. (2015) also attempted to demonstrate that the *Chlorella* species has been widely misclassified when using traditional morphological classification schemes, and suggested that only three 'true' spherical species belong to this genus: *Chlorella vulgaris*, *C. lobophora*, and *C. sorokiniana*. Based on biochemical and molecular data, the *Chlorella* genus was even more recently proposed to consist of five "true" Chlorella species (Zou et al., 2016). The number of *Chlorella* species appears to have reached ~14 with the inclusion of several former Dictyosphaerium strains (Bock et al., 2011), with suggestions of still others possible ones (Zou et al., 2016).

The submitters provided the following information to support the assignment *Parachlorella* sp. to their environmental isolate:

> "As part of the process for confirming the correct taxonomic basis for STR00010, we used the nucleotide sequence of the nuclear 18S SSU rRNA, a common phylogenetic marker, to aid in substantiating our strain as belonging to phylum Chlorophyta, class Trebouxiophyceae, order Chlorellales, family Chlorellaceae, genus *Parachlorella*. To place STR00010 in the context of other known *Chlorella* strains, we created a phylogenetic tree based on the analysis of 18S rRNA gene sequences. We selected the 18S rRNA sequences that were previously included in the published analysis of the *Chlorella* NC64A 18S rRNA gene plus the top blast matches to STR00010 rRNA sequence (*Chlorella* strains KAS012, SAG211-18, MBIC10088). The phylogenetic grouping suggests that STR00010 is part of the Parachlorella clade and is divergent from the so-called "true *Chlorella*" clade. While specific phylogenetic relationships continue to be refined, the genus *Parachlorella* was shown to be a sister phylogenetic clade closely related to the "true" spherical *Chlorella*." The resulting phylogenetic tree is shown in Figure 1.

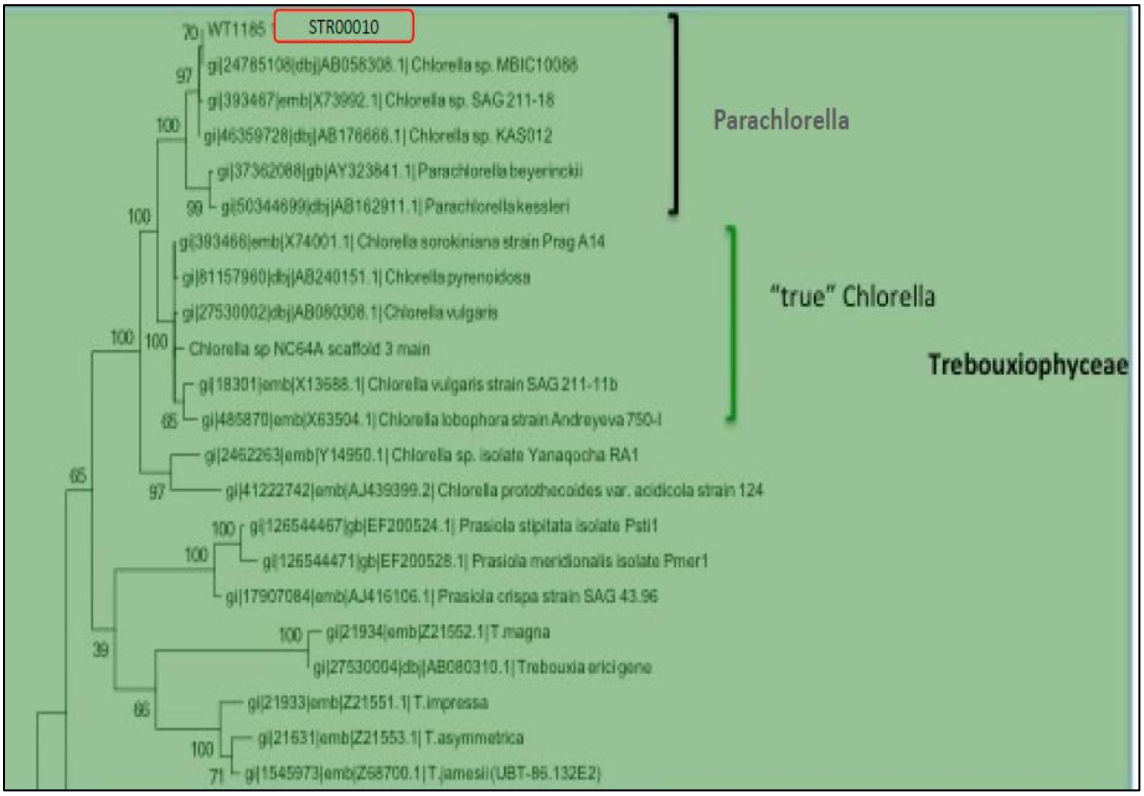

Figure 1. *Parachlorella* sp. STR00010 phylogeny (taken from TERA submission R-19-0001).

2. The Species

As previously mentioned, within the genus *Parachlorella*, there are currently two accepted species, *P. beijerinckii* and *P. kessleri* (formerly known as *Chlorella beijerinckii* and *Chlorella kessleri*, respectively). *P. hussii* has been proposed by Bock et al. (2011) and is listed in AlgaeBase (http://www.algaebase.org), but still has an uncertain taxonomic status. The morphological features of these species were described in Buxser (2019), along with closely related organisms (Table 1). The three *Parachlorella* species are described as solitary or colonial, and sometimes covered by a mucilaginous envelope (Krienitz et al., 2004; Bock et al., 2011). Like *Chlorella* spp., *Parachlorella* spp. are also known to reproduce by autosporulation (typically with 2, 4, or 8 autospores). Other features of *Parachlorella* spp. include a single parietal chloroplast and a broadly ellipsoid pyrenoid, which is covered by starch grains. All *Parachlorella* spp. can be distinguished by substitutions in the 18S rRNA gene sequence, as well as substitutions in the ITS2 region (Krienitz et al., 2004, 2011).

Table 1. Morphological features of Parachlorella and genetically-related organisms

Species	Morphology
Chlorella spp.	Cells spherical, subspherical or ellipsoid, single or forming colonies with up to 64 cells, mucilage present or absent. Chloroplast single, parietal, pyrenoid present, surrounded by starch grains. Reproduction by autospores, zoospores lacking. Autospores released through disruption of mother cell wall. Daughter cell can remain attached to remnants of mother cell wall and form colonies with mucilage envelopes. Planktonic, edaphic or endosymbiotic.
Parachlorella spp.	Solitary planktonic or edaphic globose or egg-shaped cells, sometimes with a thin, membranous gelatinous coating; parietal chloroplast with broadly ellipsoidal pyrenoid covered by starch grains; reproduction via 2, 4, 8, or 16 autospores; distinguished from other genera in the family by 18S rRNA and ITS2 nucleic acid sequences.
Parachlorella beijerinckii	As above with cells 2.5-5 x 3-8 µm with a 2-4 µm think gelatinous coat; vegetative cells are spherical or ellipsoidal with 5-8 µm diameter; single pot- or saucer-shaped chloroplast with broadly ellipsoidal pyrenoid covered with 2, 3 or 4 large cup-shaped starch grains; one or two thylakoids traverse the pyrenoid; reproduction by 2, 4 or 8 autospores sized 2.5-3.5 x 3-4.5 µm which were liberated by a broad opening in the mother cell leaving a cup-shaped empty mother cell wall remnant; cells surrounded by amorphous mucilage; electron microscopy revealed a single-layer cell wall; species differentiation by nucleic acid sequencing.
Parachlorella kessleri	In contrast to *P. beijerinckii, P. kessleri* has a mantle-shaped chloroplast and no mucilaginous coat.
Parachlorella hussii	Solitary, planktonic cells with, oval young cells and spherical to slightly oval adult cells 4.5–6.5 (7.5) µm; adult cells are surrounded by a gelatinous coat 1–3 µm thick; a single, parietal, cup-shaped chloroplast and a broadly ellipsoid pyrenoid, which is covered by two starch grains; reproduction by autosporulation with 2, 4 or 8 autospores; species differentiation by nucleic

	acid sequencing.
Closteriopsis acicularus (in *Parachlorella* clade)	Long needle-shaped with 2 to 6 starch-covered pyrenoids.
Dicloster arcuatus (in *Parachlorella* clade)	Two-celled coenobia with elongated ellipsoidal cells and long pointed apices; a single parietal chloroplast with two pyrenoids.
	Table from Buxser 2019

The parental strain used in this TERA, *Parachlorella* sp. STR00010, along with the derived strains (recipient and subject), were described as being phenotypically and morphologically consistent with a *Parachlorella* assignment. They grow as small (2-3 µm in diameter) unicellular, spherical cells (Figure 2 in R-19-0001).

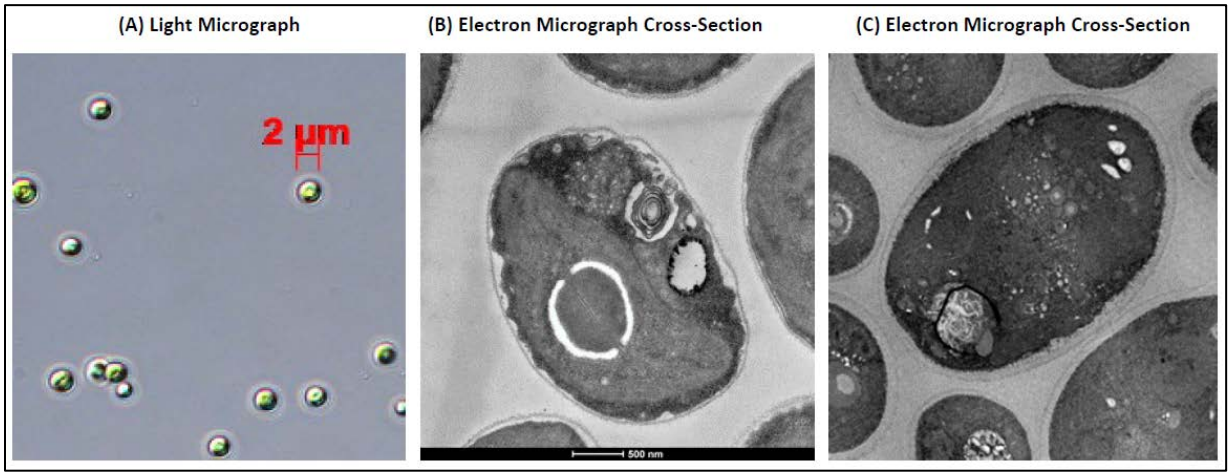

Figure 2. *Parachlorella* spp. are single celled non-flagellates microalgae (taken from TERA submission R-19-0001).

B. Donor Microorganisms

The subject strain, STR26155, is engineered to express a TurboGFP for monitoring in the environment. TurboGFP is an "improved" variant of the ppluGFP2 originally isolated from the copepod *Pontellina plumata* (phylum Arthropoda; subphylum Crustacea; class Maxillopoda; subclass Copepoda; order Calanoida; family Pontellidae) (Shagin et al., 2004). This Copepoda specimen was specifically found in samples collected in the Gulf Stream, 120 miles east of Charleston, S.C. (Shagin et al., 2004).

1. TurboGFP

GFPs from various sources have been utilized as a reporter protein and well characterized in many host systems, with minimal impact to their phenotype (Shagin et al., 2004).

The original "wild-type" version of TurboGFP, ppluGFP2, was identified and cloned along with other GFP and GFP-like proteins from Copepoda (Shagin et al., 2004). The name "TurboGFP" was later termed by Evdokimov et al. (2006) after they created an improved variant of ppluGFP2, where the maturation time was decreased, along with its tendency to aggregate *in-vitro*. Evrogen (Evrogen Joint Stock Company, Moscow, Russia) then utilized a previous codon-optimization strategy developed by

Haas et al. (1996) to allow for overexpression in mammalian systems (still retaining successful expression in many other systems), expanding its usage as a reporter protein. This TurboGFP was purchased from Evrogen by SGI and used for the current TERA.

2. loxP site

A single loxP site remains in the genome of the subject microorganism STR26155. The loxP site (34 bp sequence originally from bacteriophage P1) was part of the CRE-lox system, a cloning strategy used by the submitters to remove a selectable marker gene. This remaining sequence is non-coding and serves no function in the final subject strain STR26155.

C. Submission Microorganism

The TurboGFP gene is expected and was shown by the submitter to have no discernable phenotypic differences in the subject strain STR26155 relative to the recipient strain STR00012. Various growth tests were performed to ensure that the subject strain has no greater propensity to impact primary productivity than the recipient strain. This was proven to be true and shown in Figure 3 below.

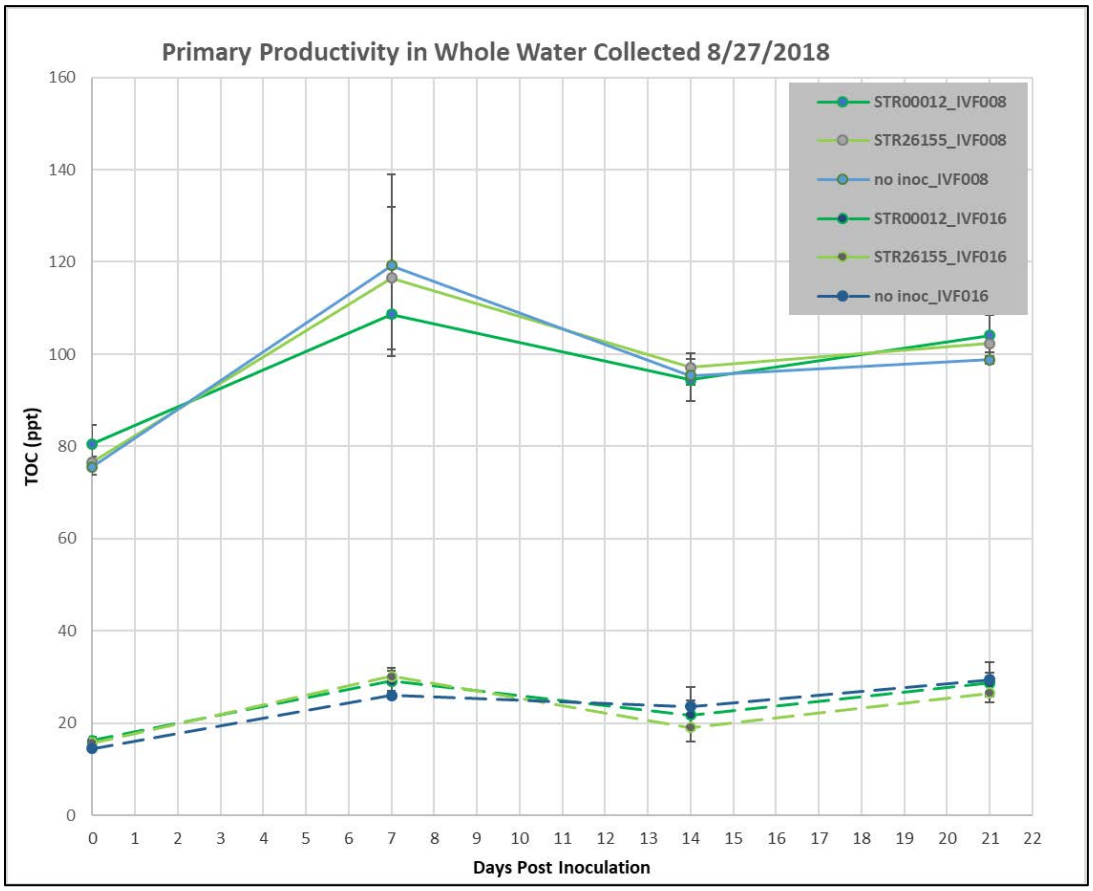

Figure 3. Whole-culture primary productivity (as Total Organic Carbon) measurements for waters spiked with recipient Parachlorella STR00012 (dark green), subject strain STR26155 (light green), and negative controls (blue) in unfiltered local waters. Waters collected from two sites, Salton Sea (IVF008, solid lines) and IID managed marsh (IVF016, dotted lines) on 8/27/2018. (Figure F8 from R-19-0001)

The submitter also conducted a detailed photophysiological comparison of the recipient and subject strain and the data are shown in Table 2.

"Biological duplicate cultures were acclimated to low light conditions prior to photo-phenotyping. Measurements were made of the maximum quantum yield of photochemistry in PSII (as Fv/Fm), functional absorption cross-section of PSII, light-saturated electron transport rate, Pmax by 14C incorporation, as well as chlorophyll (Chl) a and Chl b content of cells."

They concluded that there was no significant difference between recipient and subject strains as for all measures the differences between strains were less than the error of the measurement (CVs typically less than 5%). This experiment verified the absence of any photophysiological differences between the strains.

Strain#	FIRe (JTS-10)			^{14}C	Chlorophyll	
	F_V/F_M	σ_{PSII} (Å^2, @ 530 nm)	$1/\tau'_{Qa}$ (s^{-1})	P_{max} (nmol ^{14}C/ mg Chl/ hour)	(Chl a/TOC, %)	(Chl b/TOC, %)
STR00012	0.706 (0.010)	98 (2)	63 (2)	240 (1)	4 (0)	1 (0)
STR26155	0.708 (0.010)	101 (1)	64 (4)	238 (5)	4 (0)	1 (0)

JTS-10 parameters	Description
Fv/Fm	Maximum quantum yield of photochemistry in PSII, measured in a dark-adapted state (dimensionless). This parameter characterizes the efficiency of primary photosynthetic reactions.
σ_{PSII}	Functional absorption cross section of PSII (Å^2) in a dark-adapted state. The parameter is the product of the optical absorption cross section of PSII (i.e., the physical size of the PSII unit) and the quantum yield of photochemistry in PSII.
$1/\tau'_{Qa}$	Light saturated rate of electron transport on the acceptor side of photosystem II. This parameter indicates efficiency of linear photosynthetic electron transport

Table 2. Photophysiological characterization and comparison of recipient and subject strains. Errors are given in parentheses. (Table A1 from R-19-0001)

III. HISTORY OF USE

As stated previously, although the *Parachlorella* genus has not been assessed by US EPA, the closely-related *Chlorella* genus however, have been accessed in two previous TERA applications (R-17-0002, R-18-0001). Both *Chlorella* and *Parachlorella* are taxonomically classified in the Class Trebouxiophyceae and under the Family Chlorellaceae (Huss et al., 2009).

Chlorella (which used to include members now identified as *Parachlorella*) has a long history of research and experimentation, as it is a genus that can be found in marine, freshwater and edaphic habitats; making it one of the most ubiquitous and famous microalgal genus worldwide. Much of what

was first discovered about the fundamentals of photosynthesis and inorganic nutrition came from experiments using *Chlorella* (Shihira and Krauss, 1965).

Various *Chlorella* (*including Parachlorella*) species, have been extensively researched for their application in feed, food, nutritional, cosmetic, pharmaceutical and biofuels (Kang et al., 2004). *Chlorella* is not only a good genus for basic research but also a powerful superfood and has been proposed as a significant player in the development of second-generation biofuels and medical treatments (Kumar et al., 2016; Pienkos and Darzins, 2009). The genus *Parachlorella* specifically has been used in aquaculture as food for several shrimp species, including shrimp that is ultimately sold for human consumption (Ueno et al., 2016).

IV. GENETIC MODIFICATIONS

1. TurboGFP

The subject microorganism has an improved variant of the green fluorescent protein from copepod *Pontellina plumata* (CopGFP a.k.a., ppluGFP2; GenBank #AY268072). The vector encoding this improved variant termed TurboGFP, was obtained from Evrogen (Evrogen Joint Stock Company, Moscow, Russia). An overview of the genetic modification steps was provided by the Genetic Construction Report and presented below (Cameron, 2019).

In brief, the recipient strain was co-transformed with the *PacI*-digested NAS14335 plasmid (both the vector backbone and the fragment containing the *TurboGFP* cassette) and an active Cas9-ribonucleoprotein complex that contained a single guide RNA targeting cleavage at the genomic *RS1* locus. Per the TERA, the *RS1* site was selected with the aid of both genome and transcriptome data. The site was chosen because it was a larger intergeneric (i.e., "between genes" in this context and not indicating presence of genomic DNA from a different genus) region with no detectable transcription. The submitter's goal was to minimize the chance of unintentionally disturbing the function or regulation of nearby endogenous genes due to integration.

The vector backbone (from the commercially available pCC1BAC from Epicentre) which contained a gene that encodes resistance to the antibiotic chloramphenical (*CmR*), several other genes (*HIS3* marker, *sopABC/parABC)*, and an automatic replication sequence-yeast centromere element (ARS-CEN), among other aspects, was expected to drop out as it cannot replicate in *Parachlorella*. Its absence in the subject strain was confirmed by sequencing.

The plasmid fragment containing the *TurboGFP* cassette carried the intergeneric sequence for the resistance gene *ble* that encodes resistance to the bleomycin family antibiotics (e.g., zeocin), the *CRE* gene and associated *loxP* sites (for the Cre-lox system), as well as the *TurboGFP* cassette. This system targeted insertion of the *PacI* fragment with *TurboGFP* in the recipient strain's *RS1* site.

After co-transformation of the *PacI*-digested plasmid and the active Cas9 nuclease ribonucleoprotein (RNP) complex, transformants were selected on media with zeocin to ensure integration of the correct *PacI* fragment and ammonium (NH_4^+) to repress CRE recombinase. This was repeated once. The integration of this fragment into the recipient's genome was facilitated by endogenous non-homologous end-joining (NHEJ). Transformants were screened via colony PCR for the

correct integration at the RS1 locus. They were also screened for GFP expression with flow cytometry. A clone with the correct profile was selected (#6).

Next, the submitters induced Cre recombinase with nitrate to excise all DNA integrated at the *RS1* site that was between the loxP sites. This included deletion of one of the two loxP sites (one remains in the subject strain). After a series of passages, a culture was plated for isolated colonies, which were analyzed for correct DNA integration and *TurboGFP* expression. Isolate 15 was confirmed to have the correct profile and was designated the subject strain, *Parachlorella* sp. STR26155. At the *RS1* site, the following DNA is integrated (5'→3'): one intrageneric *HpaI* site, one intergeneric loxP site, the *TurboGFP* cassette, and a scar (2 bp insertion).

2. Antibiotic Resistance Markers

Although antibiotic markers, encoding resistance to chloramphenicol (*CmR*) and zeocin (*ble*), were elements of the plasmids used during the strain engineering process, none were present in the final subject strain STR26155. This was by design and was confirmed by colony PCR analysis, digital droplet (dd)PCR, growth (or absence thereof) on zeocin media as appropriate, and whole genome sequencing.

V. POTENTIAL ECOLOGICAL EFFECTS OF THE RECIPIENT MICROORGANISM

According to the *Parachlorella* literature review by Buxser (2019), there are no records of adverse impacts of the genus *Parachlorella* to any terrestrial plants or animals. There are also no records of toxicity or pathogenicity of *Parachlorella* to any aquatic plants or wildlife, although there may be potential for population effects related to competition/biogeochemistry (Buxser, 2019). In a broader context, the interactions of algae in aquatic and terrestrial environments and their role in aquatic food webs were discussed in a previous risk assessment for an algal submission by McClung (2017).

A. Aquatic Ecosystems

A number of factors affect the rise and fall of algal populations in the aquatic environment including the physical factors of light, temperature, weather, water movements, flotation, the chemical nutrient status of nitrogen, phosphorus, silicon, calcium, magnesium, potassium, sulfate, chloride, iron, manganese, and other trace elements, and organic matter (Ikawa, 2004). There are a number of biological factors as well including the presence of resting stages, predation, and parasitism. The polyunsaturated fatty acids produced by algae can affect algal growth. In addition, a number of biological substances are known to be produced by algae that inhibit the growth of other algal or of zooplankton grazers, as shown by Pratt (1944; Pratt et al., 1945). Likewise, it has been shown that some algae detect "infochemical" signals from grazers and can change their morphology accordingly to try to avert predation (Lass and Spaak, 2003). Food webs in water bodies are complex and dynamic and have been shown to vary from season to season and with other perturbations of the water body. e.g., eutrophication (Lindeman, 1942; Martinez, 1991).

In terms of symbiosis, *P. kessleri* along with six other algal strains previously classified as "*Chlorella*" were able to form stable symbiotic relationships with *Hydra viridis* (freshwater polyp) (Kessler et al., 1998). Competition studies have also indicated that *Parachlorella* spp. can survive in the presence of toxic cyanobacteria (Peng et al., 2011). The submitters validated this with their own

invasion/competition type experiments using both the recipient and subject strains, showing their ability to "persist in the face of competition from indigenous species".

B. Aquatic Food Webs

Algae and cyanobacteria are the basis of the food web in both freshwater and marine aquatic ecosystems. The phytoplankton community of a typical north-temperate lake has been shown to consist of up to several hundred algal species that co-exist (Kalff and Knoechel, 1978). Phytoplankton diversity is influenced not only by the different ecological niches within a water body (e.g., benthic vs. pelagic regions), but also by a number of temporal and spatial variations in factors such as nutrient supply, temperature, dissolved oxygen, predation, and parasitism (Wehr and Sheath, 2003; Townsend et al., 1998). Nutrient supply and herbivory are thought to be the most important parameters affecting diversity changes over time. According to Wehr and Sheath (2003), the phytoplankton species composition in lake food web ecosystems is important because the 'functional properties of algal assemblages vary strongly with species composition'. Different taxa are important because features that are sometimes used to classify various species such as photosynthetic pigments, storage products, motility, reproduction, cell ultrastructure, and even DNA sequence have functional importance. For example, nitrogen fixation ability is of great functional importance but is restricted to a limited number of cyanobacteria. Also, photosynthetic pigment production is important, for instance with the red accessory pigment phycoerythrin which has an absorption maximum of 540-560 nm. The presence of this pigment broadens the photosynthetic capacity of an ecosystem by facilitating growth at greater depths (Goodwin, 1974). Autotrophic picoplankton have a strong competitive advantage under phosphorus-limiting conditions (Suttle et al., 1988; Wehr, 1989).

Diversity in the size fractions of phytoplankton is an important aspect of algal communities and thus food webs. For planktonic food webs, cyanobacteria have a dominant role in aquatic productivity. It is these smaller autotrophs that provide excreted dissolved organic compounds that provide substrates for heterotrophic bacterial growth. In addition, cyanobacteria are directly grazed by protozoa (microflagellates and ciliates). This microbially-based food web in which the major portion of autotrophic production occurs is important to the marine food webs. The microbial food web consists of those organisms that are < 1000 µm, and in freshwater benthic ecosystems consists of (presented by increasing size fraction) cyanobacteria and bacteria, followed by microflagellates, diatoms and green algae, which are then consumed by ciliates, rotifers, copepods, oligochaetes, nematodes, and then invertebrate macrofauna followed by the larger vertebrates (Bott, 1996). A complex microbial food web has bacteria and algae at the lowest trophic level, which are then consumed by protozoa and meiofauna. Meiofauna are organisms in the size range of approximately 50 - 1000 µm and includes large ciliates and metazoan (e.g., rotifers, copepods, and oligochaetes).

An important link between microbial food webs and classical food webs are with the autotrophic picoplankton (> 0.2 - 2 µm). These cyanobacteria are grazed mainly by micro-zooplankton (ciliates, flagellates) rather than by cladocerans or copepods (Pernthaler et al., 1996; Hadas et al., 1998). Size affects the sinking rate with smaller planktonic species sinking more slowly. Thus, the smaller species remain more prevalent in the euphotic zone.

C. Terrestrial Ecosystems

Algae occur in nearly all terrestrial environments on earth and are invariably encountered on and beneath soil surfaces (Metting, 1981). Acceptance of algae as bona fide soil microorganisms evolved late in the 19th century when it was recognized that certain groups were restricted to soil, including some *Chlorella* species (Shihira and Krauss, 1963; Kessler, 1976). Over 38 prokaryotic genera and 147 eukaryotic genera have been identified as terrestrial species, the majority of which are truly edaphic (i.e., soil). As expected solar radiation, water and temperature are the most abiotic factors controlling their distribution, metabolism and life histories (Metting, 1981). Biotic interactions are also important, but much well less understood. Algae play an important role in primary and secondary plant community succession by acting as an integral part of ecosystem. Algal communities living in soil have the principal function of being primary producers, nitrogen fixation, and stabilization of aggregates (i.e. can even prevent soil erosion) (Metting, 1981). Algae concentrations is soils are typically found to be between 10^3 and 10^4 cells/gram but have been reported as high as 10^8 (Metting, 1981).

D. Dispersal of Algae in the environment

As reviewed by Tesson et al. (2016), microalgae have been reported across a wide range of ecosystems, covering almost all latitudes from tropical to polar regions. Due to their relatively small size (few to 500um), microalgae are dispersed by water, air, and various biotic vectors (e.g., humans and animals) (Kristiansen, 1996; Tesson et al., 2016). These mechanisms and organisms of dispersal were discussed in a previous algal risk assessment by McClung (2017).

I. Dispersal by Water

Passive dispersal of algae by water can occur wherever there is running water between connected water bodies. A study by Atkinson (1988; as cited by Kristiansen, 1996) found that the colonization of a newly constructed reservoir was from the inflow. It was several years later before the appearance of organisms other than those found in the catchment area. Heavy precipitation and flooding can result in algal dispersal by connecting water bodies that are usually isolated. Algal dispersal by water is likely more important in wetter environments that in arid regions.

II. Dispersal by Aerosols

Air is an important dispersal mechanism of algae, and it is thought that algae have spread throughout the globe as aerosols. As early as 1844 Ehrenberg recognized the presence of airborne algae in dust samples collected 300 km off the nearest coast by Darwin in 1939 on the H.M.S. Beagle (as cited by Kristiansen, 1996).

According to a review article by Sharma et al. (2007), "In general, bioaerosols range from 0.02 to 100 µm in diameter and follow the same physical rule as any particle of a similar aerodynamic diameter. They disperse via air movements and settle according to the settling velocity, available impaction, surface, and climatic factors prevailing in the area (Burge and Rogers, 2000). Air movements within a laminar boundary layer surrounding the source usually release such particles. Many of the particles remain in the layer and eventually settle near the source (<100 m), while some are carried aloft with turbulence and transported by the wind over a long distance. The processes responsible for the release and atomization of bioaerosols from natural sources are as follows:

1. Sweeping of the surface or rubbing together of adjacent surfaces by wind and gusts dislodges the bioparticles from the surface. Dried algae caught by the wind are carried away like dust particles (Grönblad, 1933; Folger, 1970).

2. Formation of oceanographic aerosols by wave action and the bursting of bubbles at the water-air interface (Woodcock, 1948; Stevenson and Collier,1962; Maynard, 1968; Schlichting, 1974). Fragments of scums and foams with algal contents along the shoreline of water bodies can be picked up by the wind and carried aloft (Maynard, 1968).

3. During heavy rainfall, algae are splashed up by raindrops and can be entrained into the atmospheric air by thermal winds (Burge and Rogers, 2000).

4. Storm activity over land and sea where great turbulence is experienced.

5. Human activities, such as agricultural practices, construction and maintenance practices, sewage treatment plants (Mahoney, 1968, as cited in Sharma et al., 2007), garbage dumping, highway traffic, and to a limited extent weapons testing and spacecraft launching, can result in the atomization of constituting algae (Schlichting, 1974; Kring, 2000).

6. Atomization of aerosols to a low height also occurs when surface water containing blooms is used for irrigation and recreational activities like boating, jet skiing, and so forth. (Benson et al., 2005)".

Sharma et al. (2007) also stated, based on the result of earlier publications, that green algae, cyanobacteria, diatoms, and tribophytes comprised most of the aero-algae flora. Cyanobacteria dominate the aero-algae flora of tropical regions whereas chlorophytes (green algae) dominate in the temperate regions.

Brown (1964) conducted studies on airborne algae using agar petri dishes suspended in stationary locations in Texas, and impaction studies of algae onto agar petri dishes from moving automobiles in 14 states. He also collected samples from an airplane. The impaction from the moving automobiles and planes yielded the greater numbers and diversity of algae. For example, the agar plates held from a moving car in Pennsylvania yielded 140 algal impactions composed of approximately 25 different genera of algae. A 10-second exposure obtained from a moving car sampling a local dust cloud resulting from plowing of a field recorded 5,000 algal compactions, of which 4,500 were chlorophycean or xanthophycean. *Chlorella* was one of the algal genera most frequently found, both in stationary dishes and impaction either by car or plane. The author stated that a large number of different genera and species can be transported in the air. The algal content of dust was quite high at > 3000 cells per m^3. The author concluded that soil is the predominant source of airborne algae.

Schlichting (1969) conducted studies on airborne algae in Michigan and Texas using Millipore filters and bubblers containing soil-water extracts at heights of 6, 15, 30, 75, and 150 feet from the ground. Also, aerial sampling of maritime algae was made from a ship 100 miles off the coast of North Carolina. Over an eight-year period, the number of algae collected never exceeded 8 /ft^2. He then estimated that a person at rest would inhale 240 algal cells per hr, which would result in an inhalation exposure of approximately 2880 cells/day. Higher algae numbers were found in the Texas samples from dust than those from water environments.

The diversity and abundance of airborne green algae and cyanobacteria on monuments and stone art works in the Mediterranean Basin was studied by Macedo et al. (2009). Airborne *Chlorella* species were found in the top three frequently encountered chlorophyta isolated which were *Chlorella, Stichococcus,* and *Chlorococcum.*

The diversity of aeroalgae in a Mediterranean river-reservoir system was found to be high (Chrisostomou et al., 2009). They found that nanoplanktonic algae comprised the majority (46.4%) of the aero-algae flora. The predominant alga was the green alga *Chlorella.* Three of the most frequently isolated nanoplanktonic airborne algae were *Chlorella vulgaris, Didymocystis bicellularis,* and *S. obliquus.* The authors suggested that these vegetative cells have a protective external coating that allows them to resist desiccation in bioaerosols for short distances.

Genitsaris et al. (2011) did a comprehensive review of studies in the published literature on airborne algae. They summarized that the most frequently occurring algae isolated from aerosols were *Chlorella, Scenedesmus, Chlorococcum,* and *Klebsormidium,* and the cyanobacterium *Lyngbya.* These were found in more than 40% of the sites that had been sampled by various researchers in their aero-algae studies.

In aquatic habitats, microorganisms are known to be concentrated in the surface films and in foams on the water surfaces (Maynard, 1968). Schlichting (1974) conducted studies on the ejection of microorganisms into the air with bursting bubbles. He found that bubbling air through a bacterial culture resulted in 2,000 times more bacteria in the bubble jet droplets. Microorganisms in the range of 0.3 to 30 μm in diameter can be carried in atmospheric water droplets (Woodcock, 1948, as cited by Schlichting, 1974).

Airborne algae are subject to desiccation stress and ultraviolet light exposure (Sharma et al., 2007). Desiccation, the equilibration of an organism to the relative humidity of the surrounding atmosphere, is an intensive stress that typically, most phototrophic organisms cannot survive (Holzinger and Karsten, 2013). However, there are studies that suggest that some algae can survive desiccation stress (Evans, 1958, 1959; Schlichting, 1961). A comprehensive list of algae capable of surviving desiccation was published in 1972 by Davis. Parker et al. (1969) reported that various cyanobacteria and green algae survived desiccation as viable algae were found in decades-old air-dried soil samples. This is in contrast to Schlichting (1960) who reported survival of only four hours with desiccation stress. Ehresmann and Hatch (1975) studied the effect of relative humidity (RH) on the survival of the unicellular eukaryotic alga *Nannochloropsis atomus* and the prokaryotic alga *Synechococcus* sp. Viable cells of the latter species could be recovered at all the RHs tested (19,40,60,80, and 100%). However, there was a progressive decrease in the number of viable *Synechococcus* cells with lower RHs. There was a stable survival at RH 92% and above. The results with the eukaryotic green alga were very different. No viable cells of *N. atomus* were recovered below 92% relative humidity. In an earlier study Schlichting (1971) found that algae remained viable under a wide range of environmental conditions including RHs of 28-98%. The stress associated with atomization of the algae was responsible for rapid decrease in viability. So perhaps, the gradual air-drying of soil samples as in Parker et al. (1969) did not result in death of the microorganisms.

Recent work by Szyjka et al. (2017) has demonstrated that cultivation of genetically engineered (GE) algae in outdoor ponds can leads to the aerosol release of these organisms. Their data shows that algae grown in ponds can travel and be detected in bucket traps as a function of distance and wind direction. Using qPCR to detect both wildtype and the GE strain showed detectable levels in all traps at

distances from 5-50 meters away. However, neither strain was able to outcompete local or airborne algae taxa in either the trap buckets or in experiments conducted using local eutrophic and oligotrophic lake water containing local taxa. Their research also showed that airborne algae have high diversity (species detected using ITS2 primers) and can invade any available waters, including members of the species being tested. This only reinforces the conclusion that aerophilous algae, such as *Chlorella*, can and will travel, both short and possibly long distances when grown in open ponds, and potential risks lie in an alga's ability to survive, establish and persist in the receiving environment. Additionally, the potential for horizontal gene transfer of the GE strains optimized genes is possible, as this same species or close relatives of this species, may be found in the surrounding environment, in both terrestrial and aquatic environments.

III. Dispersal by Aquatic and Terrestrial Organisms

Aquatic and terrestrial organisms are responsible for algal dispersal. Even fish can act as vectors. For example, numerous species of plankton algae including cyanobacteria, green algae, and diatoms have been found to pass undamaged through the digestive track of the plankton-eating gizzard shad (Velasques, 1940 as cited by Kristiansen, 1996b). Insects such as beetles have been found to carry viable algae in their digestive tract (Parsons et al., 1966, as cited by Kristiansen, 1996), and thus, their faecal pellets can distribute algae to new water bodies. Milliger and Schlichting (1968) found 20 species of green algae in the intestinal tract of beetles. Algae dispersal by beetles is a likely mechanism for small water bodies for short distances (Kristiansen, 1996). Other insects can disperse algae to various water bodies. Reville et al. (1967) found that with four species of aquatic Diptera (craneflies and midges), 21 different genera of algae were found on the collected insects. Likewise, Sides (1968) found that the mud dauber wasp was capable of carrying algae and protozoa as nine and four genera, respectively, were isolated from aseptically collected insects. Parsons et al. (1966, as cited by Kristiansen, 1996) reported the presence of 20 genera of viable blue-green algae (currently cyanobacteria), green algae, and euglenoids in and on dragonflies and damselflies. Dragonflies are thought to be able to transport algae possibly long distances (Maguire, 1963).

Water-living mammals and other mammals such as mink, muskrats, and raccoons can transport viable algae on their fur and sometimes in their intestinal tracts. Human activities can also transport algae between water bodies. For instance, the use of felt-soled wading boots has been banned in a number of states as they have been shown to transport non-native larvae, spores, and algae between water bodies. In Vermont, the felt-soled wading boots are believed to have spread didymo, a slimy alga also called rock snot, to various rivers throughout the state. This alga forms dense mats that blanket the bottom of the stream like a shag carpet, changing pristine trout streams to a green, yucky mess, according to Shawn Good, a fisheries biologist with the state Fish and Wildlife Department (http://usatoday30.usatoday.com/news/nation/environment/2011-04-28-rock-snot-felt-sole-wader-ban_n.htm).

IV. Dispersal by Birds

Water birds are the most important vectors for algae dispersal as they can transport live algae on their feet and feathers and sometimes internally in their bills or in their digestive tract. Water birds such as seagulls have been shown to transport algae, particularly aquatic desmids, in wet mud on their feet for long distances (Strøm, 1926 as cited by Kristiansen, 1996). Desiccation is of course of great importance with the viability of live algae transported on the feathers or feet of birds. Algae carried internally in the digestive tract are not subject to desiccation stress.

Migratory birds have a significant role in the transport of algae for long distances. Proctor (1959) studied the carriage of algae in the intestinal tract of numerous migratory bird species obtained from playa lakes in Texas and Oklahoma. A number of freshwater algae species were found in the alimentary canal of 25 different migratory birds. Algae were found in the lower digestive tract of the pied-bill grebe, the green-winged teal, the blue-winged teal, the shoveler, the American coot, the killdeer, the dowitcher, the American avocet, the Wilson's phalarope, and the belted kingfisher. Since many species of blue-green algae (currently cyanobacteria) and green algae do not have spores or specialized resting structures, the algae were assumed to have been transported as vegetative cells. Based upon the rate of movement of the algae through the alimentary tract and the flying speed of some common migratory birds, Proctor (1959) suggested that algae could be easily transferred between lakes 100 - 150 miles apart, with much greater distances possible with cells or colonies in the caecum of the birds.

Schlichting (1960) also investigated the transport of algae on and in various waterfowl. He measured the carriage of chlorophyta (green algae), cyanophyta (blue-green algae), chrysophyta (golden algae), euglenophyta, bacteria, fungi, protozoa, and rotifers and on the feet and feathers, and in the bill and gullet, as well as in the faecal matter of 105 birds representing the following 16 species of waterfowl: black duck (*Anas rubripes*), blue goose (*Chen caerulescens*), buffie-head duck (*Bucephala albeola*), Canada goose (*Branta canadensis*), coot (*Fulica americana*), Eastern belted kingfisher (*Megoceryle alcyon*), gadwall *(Anas strepera*), goldeneye (*Glaucinetta clangula americana*), green-winged teal (*Anas carolinensis*), mallard (*Anas platyrhynchos*), redhead duck (*Aythya americana*), ring billed gull (*Larus delawarensis*), ruddy duck (*Oxvura jamaicensis*), spotted sandpiper (*Actitis macularia*), common snipe (*Capella galinago*), and wood duck (*Aix sponsa*).

The field collection experiments demonstrated that the water birds retained viable forms of algae and protozoa both externally and internally. For those organisms carried externally on the feet and feathers, the birds exposed to the air for less than four hours carried a great variety of organisms. Those exposed to air for longer periods of time had fewer viable organisms. With eight hours exposure to air, there were some organisms on the feet of birds, but a greater variety was found to be carried in the bills. The birds exposed to the air longer than eight hours yielded very few organisms. The contents from the gullets sampled produced good algal growth in culture, whereas only a few of the 163 faecal samples contained viable algae or other organisms. Viable organisms found on the waterfowl consisted of 86 species from the feet, 25 species from the feathers, 25 species from the bills, 14 species from the gullets, and 12 organisms from the faecal material.

The following species of green algae were found on the feet of the waterfowl: *Ankistrodesmus braunii, A. convolutus, A. falcatus, Arachnochloris-like cells, Arthrospira gomotiana, A. jenneri, Chlamydomonas globosa, C. mucicola, C. pseudopertyi, C. sp., Chlorococcum sp., Chlorella ellipsoidea, C. vulgaris, Chlorella sp., Closteriopsis-like cells, Dactylococcopsis acicularis, Franceia sp., Glenodinium sp., Gloeocystis gigas, Mougeotia sp., Nannochloris bacillaris, Oedogonium sp., Oocystis rorgei. Palmodictyon sp., Protococcus sp., Rhabdoderma irregulare, Rhizoclonium fontanum, Scenedesmus abundans, S. dimorphus. S. quadricauda, Scenedesmus sp., Sphaerocystis, Schroeteri, Tetraedron minimum, T. sisconsinense, Tetraedron sp.,* and *Ulothrix sp.*

The cyanobacteria found on the feet included the following species: *Anabaena affinis, Aphanocapsa sp., Aphanothece castagnei, A. nidulans, Chroococcus dispersus, C. minutus, Gloeocapsa sp., Gloeothece linearis, Lyngbya attenuata. L. limnetica, L. sp., Microcystis aeruginosa, Nostoc sp*.(?), *Oscillatoria angustissima, O. limnetica, O. subbrevis, O. tenuis, O. terebriformis, Oscillatoria sp., Pelo-*

gloea bacillifera, Phormidium mucicola, P. tenue, Phormidium sp., Plectonema nostocorum, and *Synechococcus aeruginosus.*

Although much fewer numbers of green algae, cyanobacteria, golden algae, euglenoids, protozoa, and fungi were found on the feathers and bills, *Chlorella sp.* was found in both. It was also speculated by Schlichting (1960) that some microalgae, specifically *Chlorella*, may become embedded in the matrix of larger taxa, such as *Gloeocystis*, and be able to be transported away not only far but protected for greater periods of time.

E. Ecology of the Recipient Microorganism

Parachlorella spp. have been isolated from a wide range of freshwater (also saltwater) environments worldwide, including California (proposed TERA test site) (Figure 4; Buxser 2019). Despite this worldwide prevalence of *Parachlorella* spp., there have been no reports of adverse bloom formation from this genus. Like *Chlorella* spp., *Parachlorella* spp. are very tolerant to various growth conditions including extreme temperatures, pH, salinity, high nutrient and heavy metal concentrations (Huss et al., 1999; Juarez et al., 2011; Shimura et al., 2012; Whitton et al., 2015).

Figure 4. Locations where *Parachlorella* spp. have been isolated (Buxser, 2019)

Three genera of green algae, *Chlorella, Chlamydomonas,* and *Scenedesmus* are the dominant green algae in many aquatic habitats and are frequently isolated from marine, fresh water, soils and air samples, as they can tolerate a wide range of environmental conditions (Trainor, 1998). *Chlorella* is a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). *Chlorella* is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007).

Chlorella is resistant against a number of environmental stressors related to its metabolic versatility, and thus is able to cope with shortages of nutrients and water. This genus has a high tolerance to

temperature and can easily live in both terrestrial and aquatic ecosystems. Members of the genus *Chlorella* are found in freshwater natural and artificial water habitats throughout the world (Trainor, 1998) and some species can even thrive in polar regions and hot deserts (Hodac et al., 2016). *Chlorella* have been reported from nearly all soil types, including: desert soil crusts, where it was one of the most common genera found across 4 of 7 different biomes sampled across the Namibian-Angola border (Budel et al., 2009); humic tropical soils in India, biofilms covering natural and artificial subaerial substrates and dwell in soils, and polar desert soils in Antarctica and Artic (Hodac et al. 2016). They can be also grown in wastewater and used for the removal of metals (De-Bashan et al. 2008). Phylogenetic analysis (using SSU and ITS2 rDNA sequencing) has shown their polar, temperate and tropical distribution, in addition to demonstrating that even polar isolates are closely related to temperate ones (Hodac et al., 2016). Hodac et al. (2016) concluded based on sequence similarities that *Chlorella* might be capable of intercontinental dispersal; however, they acknowledge that their actual distributions may exhibit biogeographical patterns but requires further research. Although most *Chlorella* species are naturally free-living, some are known photosynthetic symbionts, such as one species known to be a symbiont of the unicellular protozoa *Paramecium bursaria* (Blanc et al., 2010).

Microalgae, depending on specific species characteristics and culture conditions, will employ different metabolic pathways for growth. *Chlorella* (also *Parachlorella)* may be capable of growth under autotrophic, heterotrophic and mixotrophic conditions (Kim et al., 2013). Under autotrophic conditions microalgae fix CO_2 to organic matter using light energy, which results in the reduction of CO_2. Heterotrophic microalgae can grow using organic carbon a sole carbon source without the need for light. Mixotrophic microalgae can metabolize both organic and inorganic carbon using metabolic characteristics of both auto- and heterotrophs; using energy produced from organic sources for cell synthesis and storage of chemical energy converted from light energy (See Table 3). Requirements for nitrogen and phosphorus seem to also differ between all three growth types. For example, Kim et al. (2013) reported higher requirements under heterotrophic growth conditions than for auto- or mixotrophic growth conditions. Autotrophic microalgae growth has been shown to be lower than that of heterotrophic or mixotrophic types, thus making it possible and advantageous to grow microalgae at high rates in lightless conditions that match or exceed autotrophic growth.

Table 3. Energy and carbon source of microalgae by growth type (adapted from Kim et al., 2013).

Growth type	Energy Source	Carbon Source
Autotroph	Light	Inorganic
Heterotroph	Organic	Organic
Mixotroph	Light and organic	Inorganic and organic

The growth requirements of *Parachlorella*, similar to *Chlorella*, are relatively simple, and do not differ greatly from that of other microalgae (Eyster 1967; Huss et al., 1999). For example, many *Chlorella* spp. and *Parachlorella* spp. can readily grow in Bold's Basal Medium, (containing low concentrations of phosphate, nitrate, sulfate, borate, K, Ca, Mg, Na, Zn, Mn, Mo, Cu, Co, and Fe) at pH 6.8 (Krienitz and Bock 2012). As mentioned earlier, *Parachlorella* can also utilize various energy and carbon sources. *Parachlorella*'s broad distribution can be attributed to these simple growth requirements, along with its tolerance to a variety of environmental conditions, including extremes. Examples can be seen with *Parachlorella kessleri* (previously *Chlorella kessleri*) and a previously unknown *Parachlorella* isolate found downstream from the Fukushima Daiichi Nuclear Plant (Juarez et al., 2011; Shimura et al., 2012). *P. kessleri* was isolated from a mesothermal acidic pond in Argentina with a high sulfuric acid concentration (Juarez et al., 2011). The optimal growth conditions of this isolate were: pH (2.5-3), NaCl

(1-2%), temperature (34-36°C). The isolate found near the Fukushima Daiichi Nuclear Plant could grow at high temperatures and withstand a wide range of pH (3-11), along with the ability to grow in fresh or salt water (Shimura et al., 2012). *P. kessleri* was also found at a coal-fired thermoelectric plant in Brazil where growth was measured at several concentrations of CO^2: 6%, 12%, and 18% (de Morais et al., 2007).

In a wastewater adaptation study, Osundeko et al. (2014) tested the growth of *P. kessleri* and five other species from four genera, including two *Chlorella* species, in secondary-treated municipal wastewater during an 8-week period. The results of the study showed that *P. kessleri* was one of the best at acclimating to growth in wastewater, along with its efficiency in the removal of nitrogen and phosphorus (Osundeko et al., 2014).

The occurrence of many species of algae throughout the world suggests that algae can readily disperse over great distances. Studies on microalgae have shown that most species are globally distributed (cosmopolitan) but some species have more restricted distribution due to environmental factors such as temperature or humidity, and limited dispersal mechanisms (Kristiansen, 1996). In a review of data on the distribution of coccid green algae in the environment, Komárek and Comas (1984) said that the distribution is dependent on the specific environmental requirements of the taxon. They stated that "Chlorococcalean algae (*Parachlorella* and *Chlorella* belong to this group) are traditionally supposed to be organisms of cosmopolitan occurrence. Many species occur, indeed, in various regions all over the world, but, many other taxa occur in geographically limited areas, mainly in either the northern or the tropical countries".

Chlorella, and likely *Parachlorella,* has a few known predators that are of concern for open pond cultivation, among them rotifers and some bacteria. Various strategies are being investigated for loss prevention of *Chlorella* cultures (e.g., pond crashes). Many are exploring the use of biomolecule production in algae for improving their innate defense against bacteria and rotifers (Sayre et al., 2015). Sayre et al. (2015) has examined the use of various antimicrobial peptides (AMPs) to protect against rotifer and bacterial infection and its effect on algae growth, while others are looking at genetic engineering endogenous compounds that can be produced and released by the various strains to prevent infection of the cultures. Cultivation pond experiments with *Chlorella* have demonstrated that algal-associated bacterial communities shift over time, and crashes of cultures are often associated with *Vampirovibrio chlorellavorus* infection. Therefore, various groups are working to develop PCR-based tools for monitoring contaminants. The National Alliance for Advanced Biofuels and Bioproducts (NAABB), for example, has designed primers that amplify a 1500 nucleotide region of the 18S rRNA gene from three major classes of algae: Bacillariophyceae, Eustigmatophyceae, and Chlorophyceae. "These amplicons can be sequenced for definitive identification of strains, or they can be digested with a restriction enzyme to generate allele-specific fragmentation patterns for rapid, inexpensive characterization of strains and cultures. This work provides molecular tools to detect and monitor algal population dynamics and clarifies the utility, strength, and limitations of these assays. These include tools to identify unknown strains, to routinely monitor dominant constituents in cultures, and to detect contaminants constituting as little as 0.000001% of cells in a culture. One of the technologies examined was shown to be 10,000X more sensitive for detecting weeds than flow cytometry" (Sayre et al., 2015). In addition, NAABB is also looking at developing molecular monitoring tools for tracking bacteria that are associated with the cultivation of different microalgal species as a means of determining the health of the culture and mitigating pond crashes.

Although some genera in the class Trebouxiophyceae can cause harmful algal blooms (HABs), the genus *Tetraspora*, *Parachlorella* and *Chlorella* are not associated with harmful algal blooms (HABs).

The genera *Chlorella* and *Parachlorella* are not listed as a harmful species, including in UNESCO's list of harmful micro algae (webpage: http://www.marinespecies.org/hab/ visited June 2017). These genera thrive in higher temperatures than other common species in moderate nutrient loaded environments so it is known to bloom later in the year (Elliot et al., 2006; Cordero et al., 2011). Although *Chlorella* has the potential of producing dense blooms, to date there is no available literature showing that *Chlorella* blooms have caused any adverse effects (Ryther, 1954). The only references that cite a *Chlorella* bloom event (Pan et al., 2011; Li and Pan, 2013) are based on erroneous interpretation of a paper by Ryther (1954) who mentions *Chlorella* (but not in association with the observed decimation of the oyster industry on Long Island), which was attributed to eutrophication stimulated by duck farm effluents which led to blooms of *Nannochloris atomus* and *Stichoccocus sp.* So, to date, there has been no recorded HAB event associated with *Chlorella sp.*

However, one area of concern is the ability of some *Chlorella sp.* to produce chlorellin, an antibiotic-like substance that can inhibit its own growth and that of $Gram^{+}$ and $Gram^{-}$ bacteria. Older literature has demonstrated that *Chlorella* (and thus possibly *Parachlorella*) can produce substances that are inhibitory to the growth of other algae, such as *Nitzschia frustulum* (Rice, 1949). These experiments simply exposed competing algae to the exudates of *Chlorella sp.* and did not characterized the specific molecule(s) associated with the inhibitory effect. Therefore, it is possible that Para*chlorella* may be able to outcompete other species if it is able to produce chlorellin or some other inhibitory molecule.

Potential effects of Chlorella/Parachlorella spp. on terrestrial mammals

Indirect effects on terrestrial mammals can result from ecosystem-level disruptions through the establishment of novel strains of *Chlorella* in freshwater habitats. Disruptions of these freshwater ecosystems through the introduction of new algal strains could result in harmful algal blooms (HAB) (Anderson et al., 2002). HAB events can disrupt highly complex stochastic mixing and flushing patterns and increase the eutrophication potential of waterways (Anderson, 2002; Hoagland et al., 2002). Disruptions of these waterways can negatively affect terrestrial wildlife that rely on freshwater ecosystems for food or habitat. However, as noted above, there is no literature indicating that *Chlorella* (or *Parachlorella*) has ever been responsible for HABs.

There are no reports in the literature on animal infections caused by *Parachlorella*, but effects from exposure to *Chlorella* sp., although rare, have been reported leading to infection of open wounds. Pathogenic infection of tissue by *Chlorella*, known as chlorellosis, has been reported in numerous species of mammals including gazelles, sheep (both adults and lambs), cattle, dromedaries, dogs and beaver (Cordy, 1973; Kaplan et al., 1983; Le Net et al., 1993; Philbey, 2001; Haenichen et al., 2002; Quigley, et al., 2009; Ramirez-Romero et al., 2010). Documented cases of chlorellosis are rare and are typically the opportunistic infections resulting from contamination of wounds or dissemination from the gastrointestinal tract following oral ingestion of stagnant water or sewage-contaminated water (Kaplan et al., 1983; Zakia et al., 1989; Philbey et al., 2001; Haenichen et al., 2002; Ramirez-Romero et al., 2010). Effects of chlorellosis in terrestrial mammals include the formation of lesions in the skin, liver, lungs and lymph systems accompanied by a characteristically green discoloration of the affected organs (Ramirez-Romero et al., 2010). Similar to infections in humans, ingestion of *Chlorella* has been shown to result in skin sensitivity, although organismal-level effects on terrestrial wildlife as a result of this effect are uncertain (Jitsukawa et al., 1984). While the majority of cases of chlorellosis have been reported in immunosuppressed individuals, several cases indicate that chlorellosis can occur in non-immunosuppressed mammals (Kaplan et al., 1983; Philbey et al., 2001). There is limited information

available to characterize chlorellosis infections in terrestrial wildlife so there is uncertainty related to the mechanism of infection and which species of *Chlorella* are most likely to exhibit pathogenicity.

VI. POTENTIAL ECOLOGICAL HAZARDS OF THE SUBJECT MICROORGANISM

As mentioned previously, the introduction of TurboGFP is expected and was shown by the submitters to have no discernable phenotypic differences in the subject strain STR26155 relative to the recipient strain STR00012. Various growth tests were performed to ensure that the subject strain has no greater propensity to impact primary productivity than the recipient strain. GFPs, from various sources, have been utilized as a reporter protein and are well-characterized in many host systems with minimal impact to their phenotype. The TurboGFP is not expected to introduce any new hazard concerns in the subject microorganism *Parachlorella* sp. STR26155 compared to the recipient strain.

VII. POTENTIAL SURVIVAL OF THE SUBJECT MICROORGANISMS IN THE ENVIRONMENT

As mentioned previously, *Chlorella* (and probably *Parachlorella)* is one of the most dominant green algae in many aquatic habitats and can be frequently isolated from marine, fresh water, soils and air samples, as they can tolerate a wide range of environmental conditions (Trainor, 1998). As shown in Figure 4 above, *Parachlorella* too has been isolated across the globe. *Parachlorella* is also a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). *Chlorella* is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007). *Parachlorella* is also likely to be an aeroalgae due its small unicellular nature.

In addition, Tiffany (1951), defined algae into nine different groups based on preferred habitat; including edapophytes (soil algae), aerophytes (aerial algae), endophytes (living within plant tissue) and endozoophytes (living inside animal hosts), all of which are habitats in which different *Chlorella* species have been known to thrive in. Lists of soil algae have been compiled across the country and the world, showing their diverse distribution, and frequently include *Chlorella* (Metting, 1981). Soil bound *Chlorella* species appear to tolerate high levels of radiation than other more complex terrestrial life forms (Metting, 1981). Trainor (1962) was even able to show that *Chlorella* is able to survive desiccation for one hour at 130˚C. Despite their high tolerance to a variety of stressors, Metting (1981) showed that various *Chlorella* strains are negatively affected by a variety of herbicides and insecticides, and thus could be used to minimize the dispersal of *Chlorella* (potentially *Parachlorella)* cultured in outdoor ponds. Since the genus *Parachlorella* was split out from *Chlorella*, it is likely to also survive desiccation and other stressors mentioned above.

However, little research is available that directly shows that *Parachlorella* sp. STR00010/STR26155 can survive as well as many other species in the same genera, and more research is required on the wild type strain to determine the true potential for survival posed by new strain. Ultimately, the survival characteristics are not expected to change from the wild type recipient to the submission strain.

VIII. CONCLUSIONS

The recipient microorganism, *Parachlorella* sp. STR00012, was modified by the insertion of the *TurboGFP* gene (variant of ppluGFP2 from *Pontellina plumata*) to produce the submission strain *Parachlorella* sp. STR26155. This genetic modification will provide a nucleic acid signature and

corresponding reporter protein to allow SGI to track the subject strain in open-culture and the environment. This strain was developed to have virtually no discernable phenotypic differences relative to the recipient strain and is not expected to introduce or enhance any harmful traits not already found in the wild-type strain. The proposed field test with *Parachlorella* sp. STR26155 poses low hazard for the environment and its surrounding ecological systems.

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